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Revisiting the genus *Nodularia* (Bivalvia, Unionidae): Mitochondrial phylogenomics and the description of a new species

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Abstract

The genus *Nodularia* poses a significant challenge to traditional species classification and identification due to its highly convergent and variable shells, rendering it one of the most intricate groups within the family Unionidae. Fortunately, significant progress has been made by researchers in recent years regarding the species validity and the phylogeny of this group based on molecular data. However, the inadequate exploration of regional constraints and inherent limitations in research methodologies remains a crucial factor contributing to the underestimation of species diversity. In this study, a new species of freshwater mussel from the Qingshui River in Nanning City, Guangxi Province, China, *Nodularia guiensis* **sp. nov.**, is described based on shell morphology, anatomical characters, and molecular phylogenetics. Mitochondrial phylogenomic analyses reveal the following phylogenetic relationships: (((*Nodularia hanensis* + *Nodularia micheloti*) + *Nodularia dorri*) + (*Nodularia breviconcha* + (*Nodularia huana* + (*Nodularia fusiformans* + (*Nodularia guiensis* **sp. nov.** + ((*Nodularia nuxpersicae* + *Nodularia nipponensis*) + (*Nodularia dualobtusus* + *Nodularia douglasiae*)))))))). The discovery of this new taxon further enhances the diversity level within the genus *Nodularia* in China and also highlights the necessity for comprehensive surveys of unexplored regions in order to potentially unveil additional new taxa in the future.

Key Words

Cryptic species, freshwater mussels, integrative taxonomy, mitochondrial phylogenomics, *Nodularia*, Oxynaiina, Unionini

Introduction

Indochina region is one of the world's most significant biodiversity hotspots (de Bruyn et al. 2014). The complex geological and climatic history, along with diverse landforms, may be crucial factors that contribute to the formation of highly endemic species in this region and influence the geographical distribution patterns of biological groups (de Bruyn et al. 2014). Freshwater mussels (Bivalvia, Unionidae), which are significant benthic organisms possessing effective filtering capacities for their surrounding water, also exhibit remarkable species diversity (Howard and Cuffey 2006; Strayer 2008; Vaughn et al. 2008; Vaughn 2018; Graf and Cummings 2021). Currently, approximately 140 species have been documented in rivers of Indochina, showcasing exceptional endemism within tributaries of the Chao Phraya, Pearl and Mekong rivers (Bogan 2008; Zieritz et al. 2018; Bolotov et al. 2020a; Graf and Cummings 2021; Liu et al. 2022).

The genus *Nodularia* Conrad, 1853 was initially described as a subgenus of the genus *Unio* Philipsson, 1788, with *Unio* (*Nodularia*) *douglasiae* as the type species (Conrad 1853). Later, in his synopsis of freshwater mussels, Simpson (1900, 1914) elevated it to the genus level. Subsequently, due to uncertainty surrounding the validity of the generic name *Nodularia* (e.g., Liu et al. 1979; Korniushin 1998; Prozorova et al. 2005; Graf 2007; Kondo 2008; Ouyang et al. 2011; Xue et al. 2019), the type species *douglasiae* has alternated between *Unio* and *Nodularia*. Fortunately, with recent comprehensive molecular systematics studies on Unionidae, *Nodularia* has finally achieved taxonomic stability as a genuine genus under the subfamily Unioninae (e.g., Lopes-Lima et al. 2017;

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Wu et al. 2018; Huang et al. 2019; Bolotov et al. 2020b; Sayenko et al. 2021; Zieritz et al. 2021).

Historically, species identification of *Nodularia* has primarily relied on conchological characteristics such as shell shape, sculpture, size, and color (Heude 1877, 1885; Simpson 1900, 1914; Haas 1969; Liu et al. 1979; He and Zhuang 2013). However, it is well known that the overall shape of shells exhibits substantial phenotypic plasticity and convergence (Zieritz et al. 2010; Zieritz and Aldridge 2011; Inoue et al. 2013; Wu et al. 2022), which adds complexity to the process of species delimitation. In recent years, considerable efforts have been invested in exploring species-level diversity of *Nodularia* and great progress has been made with the application of molecular techniques (e.g., Klishko et al. 2018; Choi et al. 2020; Lopes-Lima et al. 2020; Sayenko et al. 2021). Currently, the taxonomy recognizes 13 valid extant species within *Nodularia* (Wu et al. 2024a; Graf and Cummings 2024), including four newly described species as documented in our previous study (Wu et al. 2024a).

With the recent discovery of new unionids species it has been suggested that the underestimation of species diversity may result from inadequate investigation, particularly in biodiversity hotspots (Huang et al. 2019; Liu et al. 2023; Wu et al. 2023), and limitations inherent in research methods that rely solely on simplistic morphological identification of mussel unionids (He and Zhuang 2013; Bogatov and Prozorova 2017). Therefore, based on our previous research, we conducted a further investigation into the diversity of *Nodularia* species in Guangxi Zhuang Autonomous Region (hereinafter referred to as Guangxi), which is located in southern China and shares borders with Vietnam as part of the Indochina biodiversity hotspot within China.

In the present study, we proposed another new species, *Nodularia guiensis* sp. nov., from the Qingshui River in Nanning City, Guangxi, China. This new species was initially identified using DNA barcoding. Subsequently, the morphological variations among congeneric species were examined through shell morphology and soft-body anatomy analyses. Lastly, a comprehensive understanding of the phylogeny and evolution of this group was achieved based on mitochondrial genomics.

Materials and methods

Specimen sampling and morphological observations

In September 2024, four tissue samples were collected from the Qingshui River (23.4538°N, 108.7849°E) in Nanning City, Guangxi Province, China, at an altitude of approximately 331 m (Fig. 1). All specimens have been deposited as vouchers at the Museum of Zoology, Shanxi Normal University (SXNU), China (voucher numbers SXNU_24090705 – SXNU_24090708).

Conchological and anatomical features were visually examined with the naked eye and under a stereoscopic microscope, including shell shape, umbo position and sculpture, shell surface sculpture, hinge structure, muscle attachment and papillae in the incurrent and excurrent aperture (Fig. 2). A Digtal caliper (DL91150, deli得力) was used to measure shell length (L), shell width (W), and shell height (H) with an accuracy of ±0.02 mm.

DNA extraction, PCR sequencing, and mitogenome assembly

In previous studies, we constructed the most comprehensive molecular datasets of the genus *Nodularia* to date (Wu et al. 2024a). Based on this, we incorporated the newly molecular data obtained in this study and reconstructed two datasets: a DNA barcoding (COI) dataset (Suppl. material 1: table S1) and a mitogenomic nucleotide dataset (containing 12 PCGs + 2 rRNA; ATP8 was excluded due to high sequence variation; Suppl. material 1: table S2).

Genomic DNA was extracted from dissected foot tissue using the TIANamp Marine Animals DNA Kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of the mitochondrial COI gene was performed using a primer pair consisting of (LCO22me2 5′-GGTCAACAAAYCATAARGATATTGG-3′ and HCO700dy2 5′-TCAGGGTGACCAAAAAAYCA-3′, ~680 bp) (Walker et al. 2007). The PCR conditions followed the TaKaRa Ex manufacturer's protocol, with an initial denaturation step at 98 °C for 10 s, followed by 35 cycles of amplification consisting of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. The final extension was performed at 72 °C for seven minutes. Amplified PCR products were purified and sequenced by Sangon Biotech (Shanghai). All obtained sequences were deposited in GenBank (Suppl. material 1: table S1).

Doubly mitochondrial inheritance in freshwater mussels involves two types of mitochondrial DNA: maternal (F-type) and paternal (M-type) mtDNA. Here, we obtained only the F-type and conducted sequencing. For mitogenome sequencing, genomic DNA quality was assessed by agarose gel electrophoresis, and approved samples were submitted to Novogene Co., Ltd. (China) for library construction and sequencing. The sequencing procedure was performed on an Illumina Novaseq 6000 platform in accordance with the manufacturer's instructions. The resulting data comprises approximately 4GB, consisting of paired-end reads with a length of 150 bp. The raw data was filtered to obtain clean reads, which were then de novo assembled using the CLC Genomic Workbench (Qiagen). The mitogenome sequence was identified from the resulting contigs using BLAST (http://blast.ncbi.nlm.nih.gov/) and then merged into a complete mitogenome using Geneious v.11 (Kearse et al. 2012). The complete mitogenome was annotated using MITOS WebServer (Bernt et al. 2013) and submitted to GenBank via BankIt (Suppl. material 1: table S2).

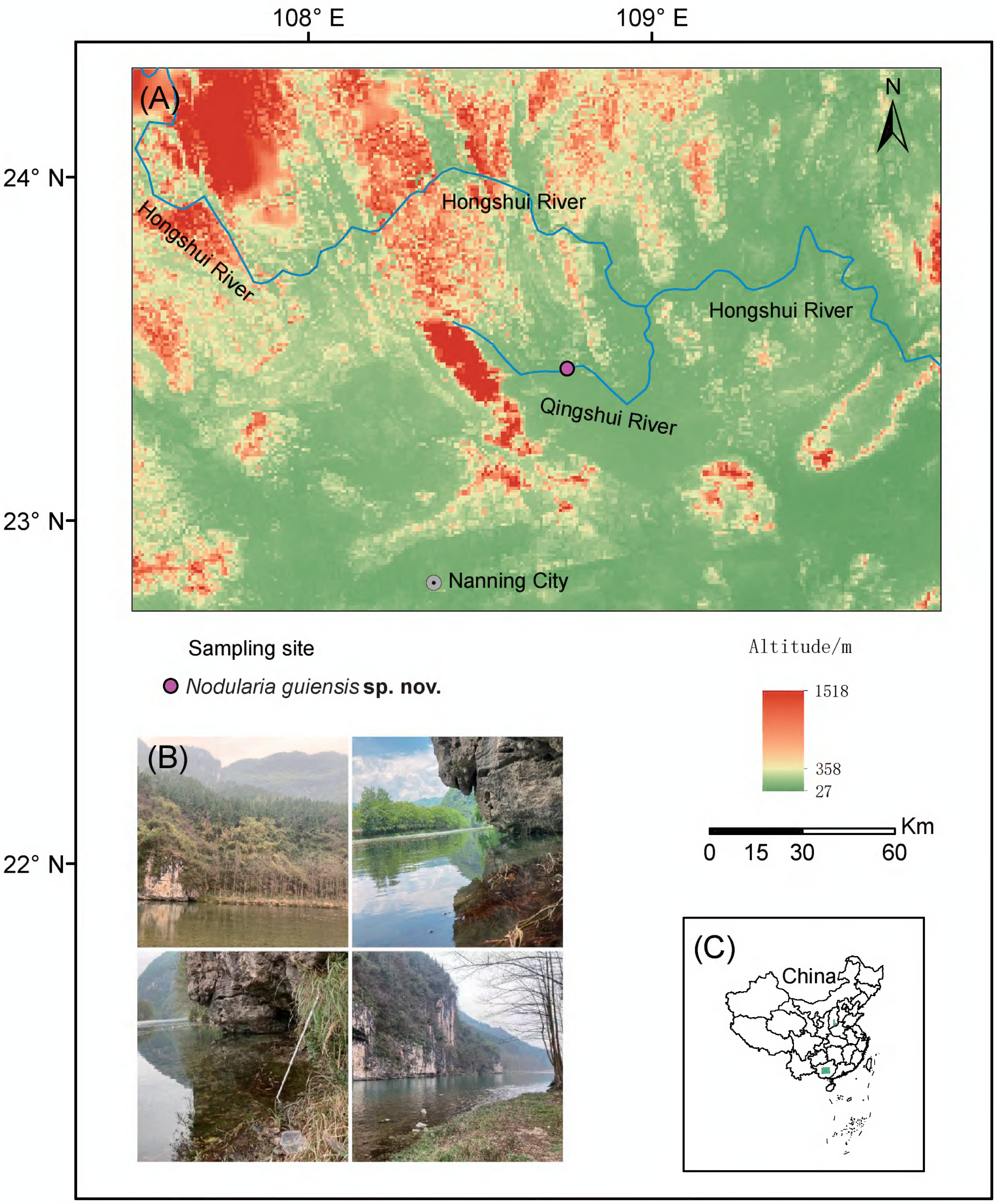

Figure 1. Map of the sampling locality and habitat of *Nodularia guiensis* sp. nov. in Guangxi Province.

Alignments and partitioning strategies

The molecular data analyses and phylogenetic reconstruction were consistent with our previous studies' methods (Wu et al. 2024a, 2024b). Protein-coding genes (PCGs) were aligned using the codon modes implemented by the built-in MACSE in PhyloSuite v1.2.3 (Zhang et al. 2020). Ribosomal genes (12S rRNA and 16S rRNA) were aligned using MAFFT v7.2 (Katoh and Standley 2013) with the L-INS-i algorithm. Ambiguous alignment areas were trimmed using Gblocks (Castresana 2000), the parameter ribosomal gene block with a minimum length was set to 2 base pairs (bp), allowed gap position was selected none; the minimum length of PCG block was set to 3 bp, allowed gap position was selected with half. The COI barcoding dataset had a fragment length of 522 bp after alignment and trimming. The mitogenomic dataset (12 PGGs + 2 rRNA) was concatenated using Phylosuite v1.2.3.

The built mitogenomic dataset was partitioned based on genes and codons using PartitionFinder (Lanfear et al. 2017) to select Bayesian inference (BI) analysis models for the partitioning schemes. ModelFinder (Kalyaanamoorthy et al. 2017) was employed in IQ-TREE (Minh et al. 2020) to determine the maximum likelihood (ML) analysis models. The selection of best-fit models was based on the corrected Akaike Information Criterion (AICc). Substitution models assigned to each partition by PartitionFinder and ModelFinder were listed in Suppl. material 1: table S3.

Genetic distances calculation and phylogenetic analyses

The intraspecific and interspecific genetic distances were computed using the COI dataset with the uncorrected *p*-distance model in MEGA 7.0 (Kumar et al. 2016).

The IQ-TREE web server (http://iqtree.cibiv.univie.ac.at/) was used for Maximum Likelihood (ML) phylogenetic analysis based on the COI dataset and the mitogenome dataset, employing the ultrafast bootstrap algorithm with 1000 repetitions (Minh et al. 2013). Bayesian inference (BI) phylogenetic analyses were conducted in MrBayes v2.01 (Ronquist et al. 2012), using models generated in PartitionFinder (Lanfear et al. 2017). Four independent Markov Chain Monte Carlo (MCMC) chains were simultaneously run for ten million generations, with sampling conducted every 1000 generations and a burn-in of 25%. The process terminated when the average standard deviation of splitting frequency dropped below 0.01. Finally, the constructed phylogenetic trees were utilized in online iTOL (https://itol.embl.de/itol.cgi) for editing and visualization (Letunic and Bork 2007).

Results

Systematics

Family Unionidae Rafinesque, 1820
Subfamily Unioninae Rafinesque, 1820
Tribe Unionini Rafinesque, 1820
Subtribe Oxynaiina Starobogatov, 1970

Genus *Nodularia* Conrad, 1853

Type species. *Nodularia douglasiae* (Griffith & Pidgeon, 1833).

***Nodularia guiensis* Hou & Wu, sp. nov.**

https://zoobank.org/9C6D0A53-C775-4B72-8A78-C800C2BBE9F7

Fig. 2

Type material. ***Holotype*:** • SXNU_24090707 (length 46.57 mm, height 21.34 mm, width 15.68 mm) (Fig. 1D); ***Paratypes*:** • SXNU_24090705; SXNU_24090706; SXNU_24090708 (Fig. 1A–C).

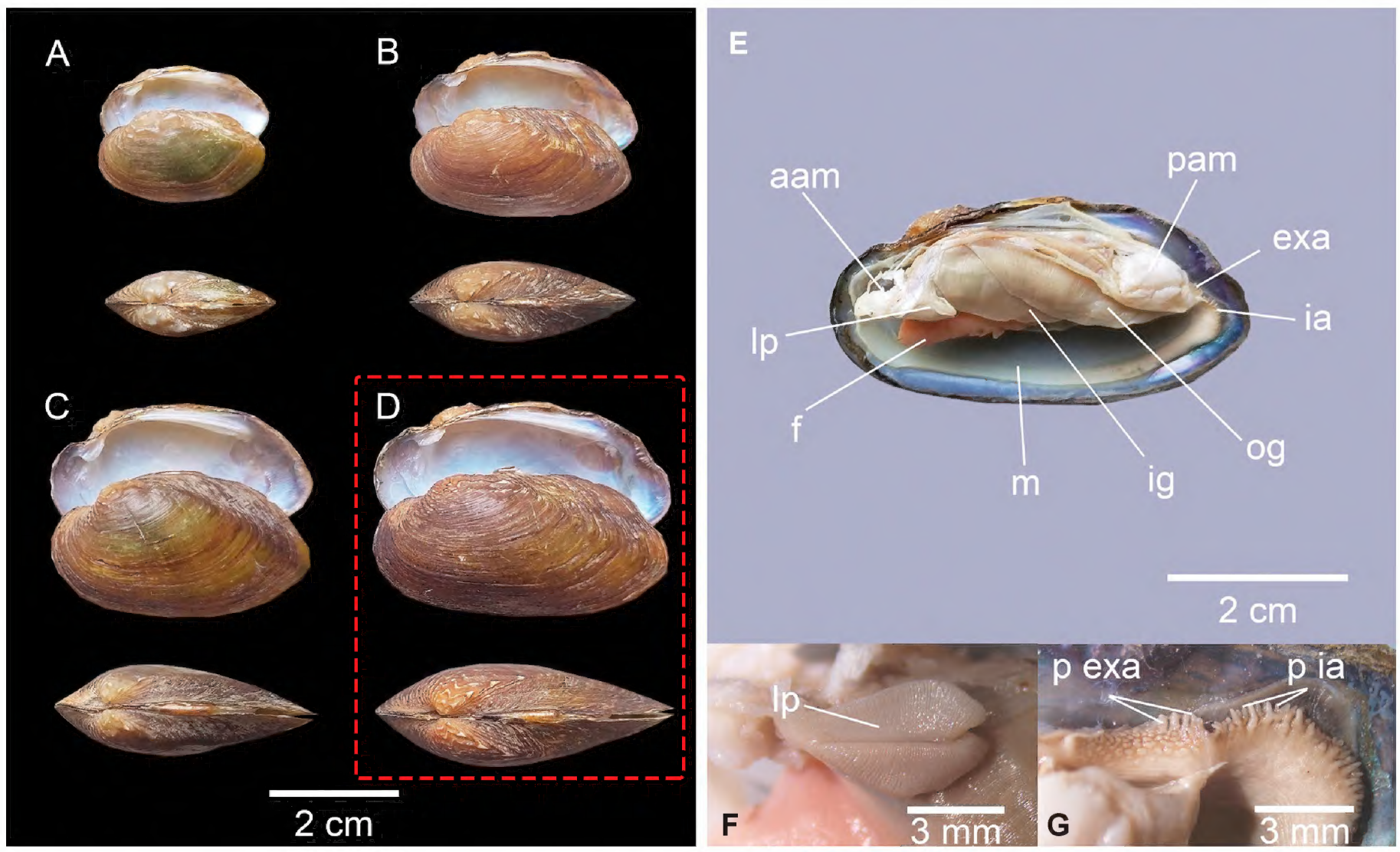

Figure 2. Shells and anatomical features of *Nodularia guiensis* sp. nov. **A–D.** Paratypes: SXNU_24090705 (**A**); SXNU_24090706 (**B**); SXNU_24090708 (**C**); Holotype: SXNU_24090707 (**D**); **E.** Anatomical features with the left valve removed. Abbreviations: aam, anterior adductor muscle; pam, posterior adductor muscle; exa, excurrent aperture; ia, incurrent aperture; f, foot; ig, inner gill; og, outer gill; m, mantle; lp, labial palp. **F.** Labial palps; **G.** Papillae in apertures. Abbreviations: p ia, papillae in incurrent aperture; p exa, papillae in excurrent aperture.

Etymology. We collected this species in Guangxi Province, so we named it 'Gui' after the abbreviation of Guangxi Province and then Latinized it. We recommend using 'Gui Jiejie Bang' (桂结节蚌) as the common name.

Diagnosis. Shell medium-sized, moderately thick, overall slender shape; anterior margin obviously indented with shell umbo resulting in lower dorsal margin. The shell morphology of this newly discovered species is highly similar to *Nodularia huana*. But the new species can be easily distinguished from other congeneric species using the COI barcode (Fig. 3). It has a genetic distance (uncorrected *p*-distance) of 7.7% from *Nodularia huana*, while its genetic distance from *Nodularia fusiformans* is the smallest at 5.5% (Table 1).

Description. Shell medium-sized, moderately thick, overall slender shape; dorsal margin curved; anterior margin round; ventral margin nearly straight; posterior margin slightly blunt, located at almost 1/2 of shell height; umbo located at 1/4 of shell length and slightly higher than the dorsal margin; umbo sculptured with rough nodes or *W*-shape ridges; epidermis yellow-brown and young epidermis light brown with a little green, shell surface covered with fine to rough concentric ridges; ligament short, narrow, and brown; anterior adductor muscle attachment elliptical; posterior adductor muscle attachment round or oval and smooth; mantle muscle attachment obvious; left valve with two pseudocardinal teeth, lamellae, anterior tooth wide and flat, posterior tooth slightly thick and pyramidal, anterior pseudocardinal tooth of some individuals split into two pieces, and having two lateral teeth; right valve with one thick pseudocardinal tooth and one lateral tooth; lateral teeth of both valves long and compressed; nacre silvery-white to bluish-white, and shiny (Fig. 2).

Anatomical characteristics. The papillae of the incurrent aperture conical, arranged in two to three rows; weakly developed papillae of the excurrent aperture arranged in one row, and knobs or raised bumps dense; the pigmentation of the incurrent and excurrent aperture absent; labial palps medium-thick (Fig. 2).

Distribution. Sanli Town, Shanglin County, Nanning City, Guangxi Province.

Phylogenetic analyses

The ML tree based on the COI dataset showed that the newly collected specimens formed an independent monophyletic group (Fig. 3). The average COI genetic distance between the specimens and the closest related *Nodularia huana* and *Nodularia fusiformans* exceeded 5% (Table 1).

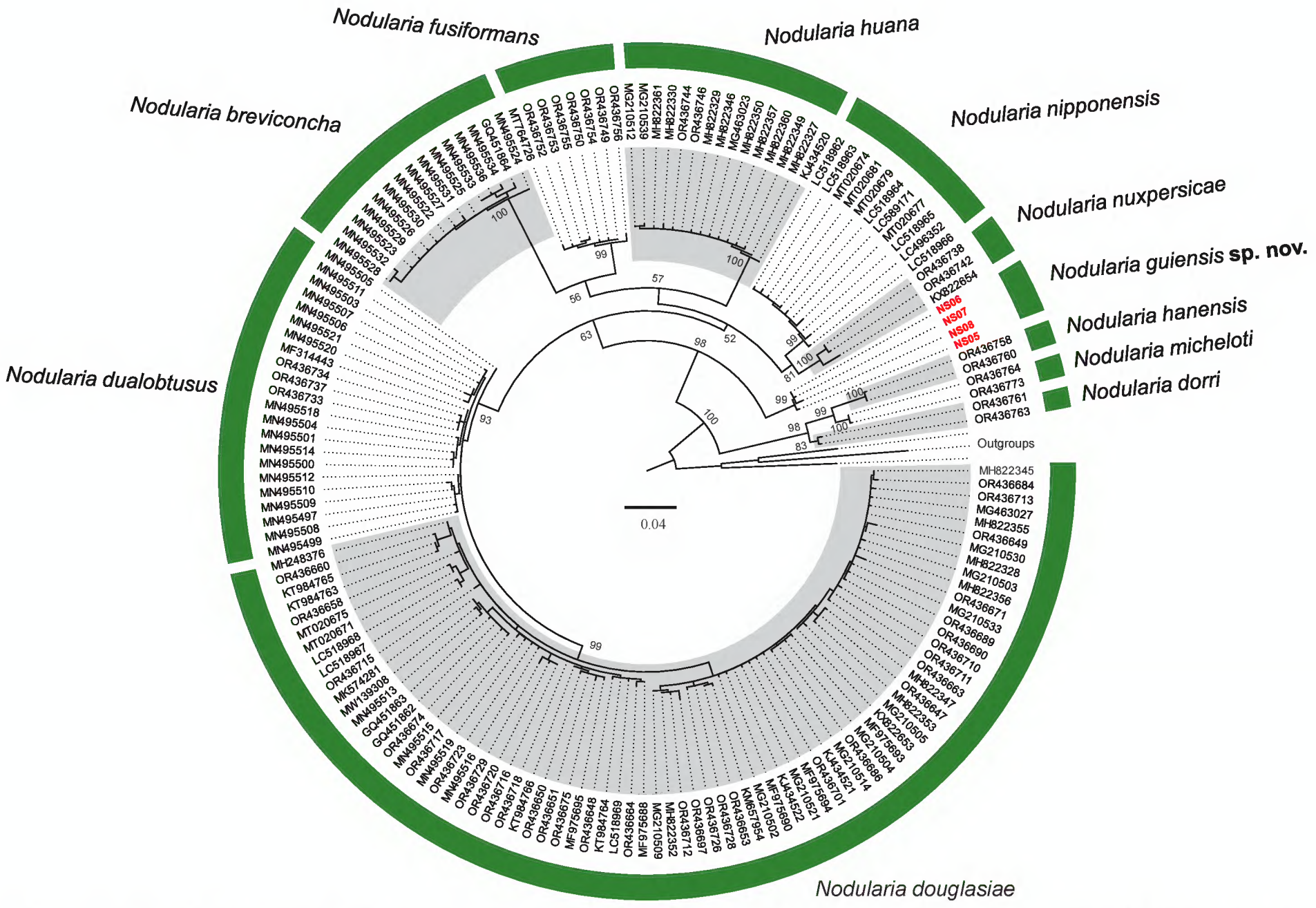

Figure 3. Phylogenetic tree of *Nodularia* species inferred by maximum-likelihood (ML) analysis based on the COI barcode dataset. Bootstrap-support (BS) values are shown at the nodes. The red specimen codes are from this study.

Table 1. Intra- and interspecific genetic distances assessed using 1000 bootstrap replicates based on the uncorrected *p*-distance model in MEGA 7.0.

	Intraspecific distances	Interspecific distances									
Nodularia guiensis **sp. nov.**	0.005										
Nodularia douglasiae	0.019	0.070									
Nodularia breviconcha	0.009	0.085	0.093								
Nodularia huana	0.004	0.077	0.084	0.086							
Nodularia nuxpersicae	0.016	0.064	0.060	0.094	0.066						
Nodularia nipponensis	0.008	0.066	0.056	0.098	0.075	0.048					
Nodularia dualobtusus	0.010	0.058	0.030	0.094	0.073	0.052	0.049				
Nodularia hanensis	0.002	0.110	0.113	0.124	0.108	0.115	0.114	0.105			
Nodularia micheloti	0.002	0.108	0.104	0.123	0.109	0.112	0.112	0.103	0.039		
Nodularia fusiformans	0.006	0.055	0.069	0.084	0.060	0.063	0.063	0.059	0.121	0.117	
Nodularia dorri	0.006	0.092	0.094	0.105	0.099	0.104	0.106	0.094	0.051	0.041	0.110

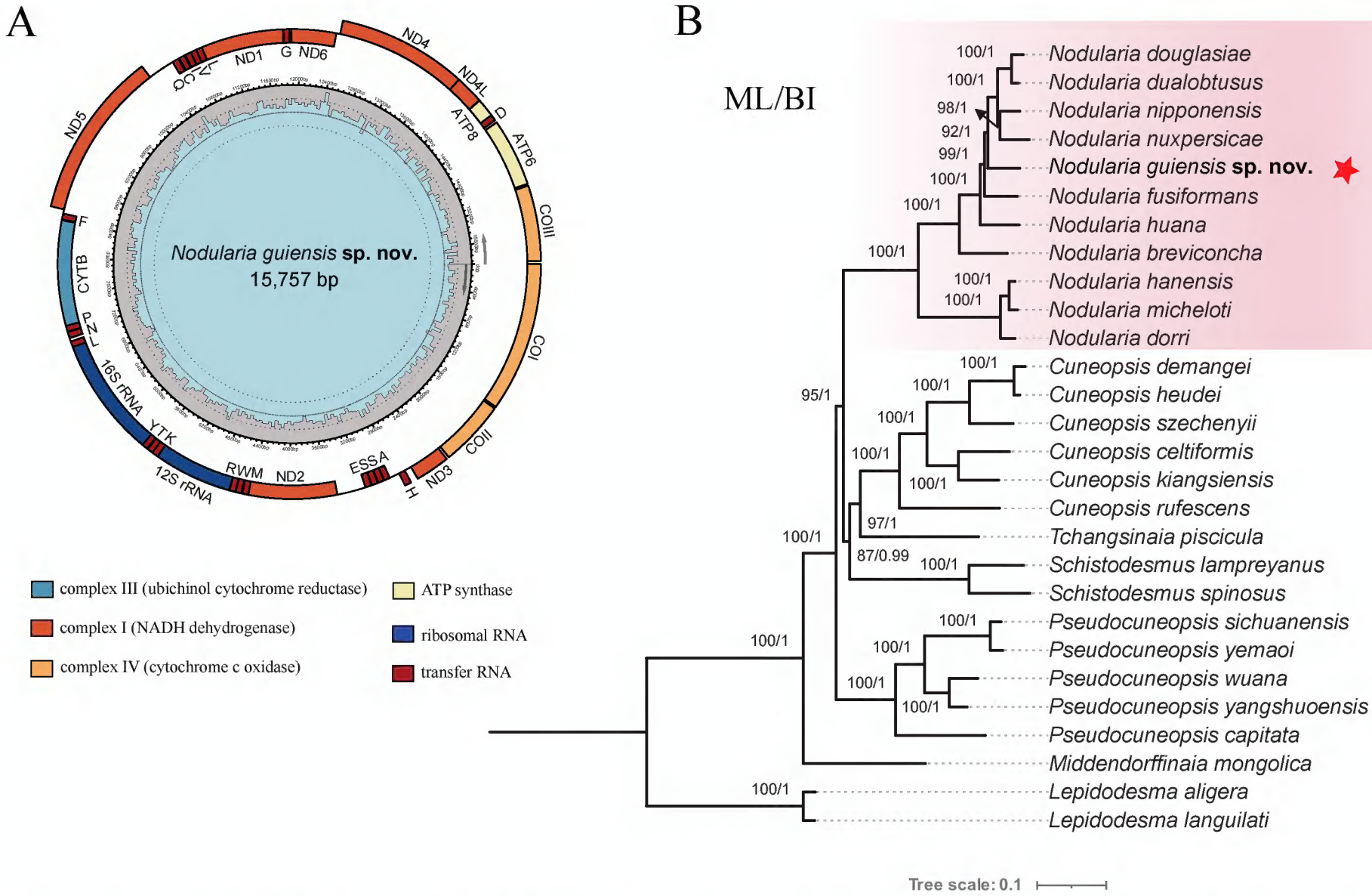

Figure 4. Analysis based on mitochondrial data. **A.** Gene map of the F-type mitochondrial genome of *Nodularia guiensis* sp. nov. **B.** Phylogenetic trees inferred from Bayesian Inference (BI) and Maximum Likelihood (ML) analyses. Support values above the branches are maximum likelihood bootstrap supports (BS) and Bayesian posterior probabilities (PP), respectively. The colored shaded clades represent the genus *Nodularia* taxa that are the focus of this study. Pentagrams symbolize the sequences from this study.

In this study, we constructed phylogenetic trees using the complete mitogenome from 26 Unionini species and two outgroups. Both ML and BI trees produced identical topologies with high support on most nodes (BS > 90%, PP > 0.9, Fig. 4). All genera within the tribe Unionini formed a robust monophyletic clade (BS > 85%, PP = 1.0, Fig. 4). Focusing on the genus *Nodularia*, both phylogenetic trees consistently supported relationships at the species-level as follows: (((*N. hanensis* + *N. micheloti*) + *N. dorri*) + (*N. breviconcha* + (*N. huana* + (*N. fusiformans* + (*N. guiensis* sp. nov. + ((*N. nuxpersicae* + *N. nipponensis*) + (*N. dualobtusus* + *N. douglasiae*))))))) (Fig. 4).

Discussion

Intraspecific morphological variation and interspecific morphological convergence in mussel unionids, particularly in *Nodularia*, pose a challenge to species identification (Inoue et al. 2013; Huang et al. 2018; Wu et al. 2022). Thus, relying solely on morphological characteristics is less robust for species identification (Zieritz and Aldridge 2009; Inoue et al. 2013, 2014; Wu et al. 2022). Recent advancements in molecular techniques have significantly contributed to the taxonomy and phylogeny of *Nodularia* (Klishko et al. 2018; Choi et al. 2020; Lopes-Lima et al. 2020;

Table 2. Conchological characters of *Nodularia guiensis* sp. nov., *Nodularia diespiter*, *Nodularia persculpta*, and *Nodularia gladiator*.

Characters	*N. guiensis* sp. nov.	*N. diespiter*	*N. persculpta*	*N. gladiator*
Shell shape	Oval to slender shape	Oval	Elliptical	Elongated
Umbo sculpture	Umbo sculptured with rough nodes or *W*-shape ridges	Corroded	Umbo sculptured with *V*-shape ridges	Corroded
Surface sculpture	Epidermis yellow-brown and young epidermis light brown with a little green; shell surface covered with fine to rough concentric ridges	Epidermis black-brown; shell surface covered with concentric ridges	Epidermis yellow with dark-green lirelliform wrinkles covering the shell surface	Epidermis dark brown; shell surface rough and decorated with concentric ridges
Nacre	Nacre silvery-white to bluish-white, and shiny	Nacre silvery	Nacre milky-white to silvery-white	Nacre milky-white
Anterior margin	Anterior margin obviously indented with shell umbo resulting in lower dorsal margin	Anterior margin obtuse	Rounded	Rounded
Dorsal margin	Curved	Anterior dorsal margin upward and almost sloping in a straight line, and the posterior dorsal margin curved	Curved	Slightly curved
Ventral margin	Nearly straight	Nearly straight	Nearly straight	Nearly straight
Posterior margin	Slightly blunt	Slightly pointed	Slightly blunt	Sharpened

Sayenko et al. 2021; Wu et al. 2024a). Currently, there are 13 recognized species of *Nodularia* (Graf and Cummings 2024; Wu et al. 2024a). This study successfully integrates morphological characteristics and molecular systematics to identify a new species from Guangxi, China, i.e. *Nodularia guiensis* sp. nov. The genetic *p*-distance between the new species and the ten currently available *Nodularia* species was computed, revealing a minimum distance of 5.5% compared to that of *Nodularia fusiformans* (Table 1). This value significantly exceeds the commonly accepted threshold of 3% for inter-species divergence (Hebert et al. 2003; Barrett and Hebert 2005). We carefully compared the morphological characteristics of the new species with those of the other three species. *Nodularia diespiter* (Mabille, 1887) and *Nodularia gladiator* (Ancey, 1881) were found in the Red River basin of North Vietnam. Both species have a distinctly sharp posterior margin and exhibit discernible morphological differences from the new species (Table 2). The shell surface of *Nodularia persculpta* Haas, 1910 is covered with clear *V*-shaped ridges, which easily distinguishes it from the new species (Table 2).

Given the severe decline of freshwater mussel species globally, understanding their phylogenetic diversity and evolutionary relationships is crucial for determining conservation priorities (Mace 2004; Lopes-Lima et al. 2017; Saito et al. 2022). Our phylogeny of the mitochondrial genome is consistent with previous studies (Wu et al. 2024a) and strongly supports the relationship between the new species and its congeneric species, providing an important foundation for better understanding its potential ecological characteristics and reproductive strategies.

The recent studies continue to reveal descriptions of novel species or new records of freshwater mussels in Guangxi, a recognized biodiversity hotspot, including the discovery of *Nodularia guiensis* sp. nov. in this study (Dai et al. 2023; Liu et al. 2023; Wu et al. 2023; Liu LL et al. 2024a). As further exploration of the region continues, it is reasonable to anticipate the discovery of unknown taxa in the future. However, this prospect raises concerns regarding the best way to conserve diversity amidst ongoing threats posed by mussel habitat degradation and fragmentation resulting from pollution and urban development prior to the discovery of these taxa (Dudgeon et al. 2006; Tockner et al. 2010; Dudgeon 2019; Liu XJ et al. 2024b). We aspire to expedite the discovery of additional taxa, which will enable a comprehensive understanding of their biological and ecological characteristics, geographical distribution, potential risks, as well as host fish information. It is like a race against time.

Acknowledgements

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Supplementary material 1

Supplementary tables

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Data type: xlsx

Explanation note: table S1: List of COI sequences used in this study, including the species, specimen codes, GenBank accession numbers, voucher specimen number and collecting locations. (*) Sequences from this study; table S2: Complete mitogenome sequences used in this study. (*) Sequences from this study; table S3: Partitioning strategies from ModerFinder and PartitionFinder for mitogenome dataset.

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